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Developmental Stages of a Smooth-Walled Filamentous Bacterium Associated with Equine Cyathostomes

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ABSTRACT: Communities of microorganisms colonize the anal and vulvar pores on the posterior extremities of female cyathostomid nematodes recovered from Burchell's zebras, *Equus burchelli antiquorum*. Cyathostomes with attached filamentous microorganisms were processed for scanning and transmission electron microscopy using standard methods. The adherence and in situ development of a filamentous bacterium, designated as a smooth-walled multicellular organism, or trichome-forming bacterium, is described. A vegetative cell complex that adheres to the cyathostome cuticle gives rise to unbranched aerial filaments. These filaments develop by means of multiple transverse and longitudinal septation to form a multicellular filament enclosed in a common cell-wall profile. New cellular units (microgonidia) may be released from mature filaments into the ingesta of the hindgut, where they attach to a cyathostome cuticle and develop new daughter filaments. This is the first known report on the development and adherence of such a trichome-forming filamentous bacterium. The significance of the structure, development, and association of this filamentous bacterium and nematode are discussed. Its exact classification is still unknown.

KEY WORDS: Burchell's zebra, equine cyathostomes, filamentous bacterium, trichome-forming bacteria, microorganisms, SEM, TEM, morphology, developmental cycle, microbial communities.

Free-ranging equids (i.e., zebras) are host to large numbers and an extensive diversity of nematodes (Krecek et al., 1987a, b). Microbial communities have been observed colonizing the anal and vulvar regions of cyathostomid nematode females without apparent pathological consequences (Krecek et al., 1987b; Mackie et al., 1989; Els and Krecek, 1990). Previous studies have described the ultrastructure and proposed developmental stages of some of these microorganisms as well as their relationship to their cyathostome hosts. Attempts have also been made to isolate and characterize some of these bacteria (Krecek et al., 1987b; Mackie et al., 1989; Els and Krecek, 1990; Els et al., 1991).

Studies on the structure and developmental cycle of a segmented filamentous bacterium (Els and Krecek, 1990), a helical bacterial filament (Els et al., 1991), and other components of these microbial communities (Mackie et al., 1989; Krecek et al., 1992) have contributed to the knowledge needed to understand these microorganism–nematode host relationships. Attempts to culture these organisms have as yet been unsuccessful. A smooth-walled multicellular filamentous bacterium constitutes the third known constituent of filamentous structures associated with cyathostomes (Els and Krecek, 1990). Although some features resemble those of other filamentous or trichomous bacteria observed in a number of animals (Chase and Erlandsen, 1976; Trentini, 1981; Savage, 1983; Hirsch, 1989; Strohl, 1989), the adherence process and developmental stages appear to be complex and unique. This report proposes some developmental phases and an adherence process of this filamentous bacterium to the cuticle of the cyathostome.

Materials and Methods

Electron microscopy

Cyathostomes were collected and processed for electron microscopy according to methods described by Els and Krecek (1990).

Microbiology

To cultivate the microorganisms and reduce contamination from hindgut flora, the nematodes were rinsed with phosphate-buffered solution and cultured in several enriched media. The media included blood tryptose agar (BTA) (tryptose agar with 10% bovine blood), BTA with 50 mg/liter nalidixic, and worm-agar, which were intended for anaerobic incubation (Krecek et al., 1992). Because the smooth-walled multicellular filament resembled some features characteristic of trichomes in the genus *Caryophanon*, attempts were also made to isolate this filamentous organism or any *Caryophanon* spp. from zebra feces. The procedure of enrichment of *Caryophanon* described by Trentini (1981) was followed.

Terminology

To avoid confusion, we used the terminology for filamentous bacteria referred to by Hirsch (1989), Sayre

and Starr (1989), Strohl (1989), and Trentini (1986). In addition, some mycological terminology was used to describe this filamentous microorganism. Such terms include macrogonidia and microgonidia to indicate the disclike and spherical propagation cells observed, respectively (Hirsch, 1989), as well as thallus (Krecek et al., 1987b).

Results

Electron microscopy

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) revealed a diverse group of microorganisms associated with the reproductive and digestive tract openings of female cyathostomes of zebras. Among these microorganisms, SEM consistently revealed a smooth-walled multicellular filamentous bacterium (Fig. 1) that showed internal septate structures apparently in various stages of development when viewed by TEM (Figs. 2–14).

Filament morphology

Filamentous bacteria varied from 1.6 to 2.3 μm (average 1.8 μm) in width and measured up to 500 μm in length. The wall, about 100 nm thick, was longitudinally continuous in appearance in thin sections. The cell-wall complex included several layers shown in transverse and longitudinal sections (Figs. 2, 3). Both light and electron microscopy revealed a type of Gram-positive structure. The outer part of the filament wall included both a moderately electron-dense fibrillar layer (F = 55 nm) and a denser inner layer (D = 20 nm). This was contiguous to a faint, moderately electron-dense layer (N = 30 nm), often resolved as a faint nonmembranous structure. An electron-lucent space (L = 15 nm) separated the outer layers from an inner layer (CM), which demarcated the discs or inner cellular compartments (Figs. 2, 3). The CM was resolved as a typical unit cell membrane or cytoplasmic membrane (CM = 8–10 nm) forming the septal walls of the enclosed disc material. When ruthenium red (RR) stain was added to the fixative, the outer layers appeared more electron-dense and their fibrillar nature was clearly observed, especially when observed in cross-section (Fig. 2). RR thus revealed a polysaccharide component present in the fibrils (Handley et al., 1988).

Developmental cycle of the filamentous bacterium

A developmental sequence of the filamentous bacterium is proposed in Figs. 4–14. Filaments appeared to develop from a vegetative growth complex that is either cauliflowerlike or random grapelike aggregates of cellular units. Each unit consisted of an outer electron-dense fibrillar structure that surrounded an electron-lucent space with a moderately dense core (Figs. 4, 5). The fibrillar layer of the units adhered superficially to the cuticle of the cyathostome without penetration. The complex stained more electron-dense with RR indicating a polysaccharide content. Unbranched filamentous structures were observed to originate from the growth complex. These filaments initially developed as single barrel-shaped cells (thalluslike) anchored to the cuticle by means of 1 or more rooting structures in the growth complex (Figs. 4, 5). From the thallus, elongation proceeded at the free end by the formation of internal undifferentiated cellular units separated by intracellular septa (Fig. 5).

At the junction of the thallus and the adjoining cell, a ring of closely arranged spherical or club-shaped structures were noted, each consisting of a clear halo around a moderately electron-dense center. The ring may be involved in the addition of cells to the developing filament (at least in the initial stage). The appearance and number of components in the ring varied according to the plane of section (Figs. 4, 5 and inset in Fig. 5). During initial cell formation, each completed cell unit within the actively growing filament also exhibited growth of secondary septa with the CM invaginating like the closure of an iris diaphragm (Fig. 6). This resulted in the formation of new discs (macrogonidia) and apparent elongation of the filament (Figs. 7, 8).

A set of septate walls that formed perpendicularly to the existing septa was the subsequent stage of development. These walls separated the macrogonidia (Fig. 9) and resulted in numerous spherical-shaped units, microgonidia (Figs. 10, 11) bound by a common cell wall (hence the designation multicellular filament). In the final stage of development, mature individual microgonidia (daughter cells) may be released from the mother filament into the hindgut environment (Figs. 12, 13) to be dispersed to new sites of attachment (Fig. 14).

Discs (Fig. 3, macrogonidia) showed homogeneous cytoplasm and nuclear areas with the appearance of prokaryotic cells. Mesosomelike structures occasionally observed were considered fixation artifacts (Hobot et al., 1985). No intrasegmental bodies (holdfast segments) simi-

lar in morphology to the initial thallus were observed in any segment or disc.

The actual membrane structures involved in the formation of the discs are shown in Figure 15. In septum formation, the external fibrillar layer remained unindented, involving only the cell unit membranes (CM) adjacent to its internal side. The CM replication at the site of septum growth gave rise to an internal budding profile of newly formed CM (S in Fig. 15). Invaginations of these membranes preceded the annular ingrowth of the future cell septa. Shortly before the process of septum formation was completed, the ingrowing CM skirting the septa joined to form 2 club-shaped structures (Fig. 15). Fusion of these shapes (with the final splitting of the replicating CM and subsequent filling with septum material) resulted in a complete transverse septum to form 2 new cells.

Microbiology

None of these culture techniques was successful with regard to the isolation of any recognizable filamentous bacteria or *Caryophanon* spp.

Discussion

Previous studies have shown that the cuticle of cyathostomes recovered from the zebra hindgut supports a large and diverse population of bacterial forms including 3 filamentous types (Krecek et al., 1987a, b; Mackie et al., 1989; Els and Krecek, 1990). Using TEM, morphological evidence of a filamentous smooth-walled multicellular bacterium or trichome-forming bacterium is presented with a detailed developmental sequence.

Ultrastructural evidence exists for developmental cycles of segmented, filamentous bacteria attached to intestinal epithelial cells in various hosts (Davis and Savage, 1974; Chase and Erlandsen, 1976; Breznak and Pankratz, 1977; Bracke et al., 1979; Savage, 1983). These bacteria differ from those in the present study by having an association with epithelial linings in various mammalian, avian, and insect intestinal tracts and the ability to form endospores or specialized holdfast cells. In contrast, in the present study the bacteria are associated with an invertebrate host inside a mammalian hindgut and a thallus (Krecek et al., 1987b) appears to be the initial generating reproductive cell.

The filamentous bacterium of the present study differs from other known members of the microbial community in the zebra hindgut in its bacterial wall structure, mode of septation, and means of adherence to the nematode cuticle (Mackie et al., 1989; Els and Krecek, 1990; Els et al., 1991). Although extracellular fibrous struc-

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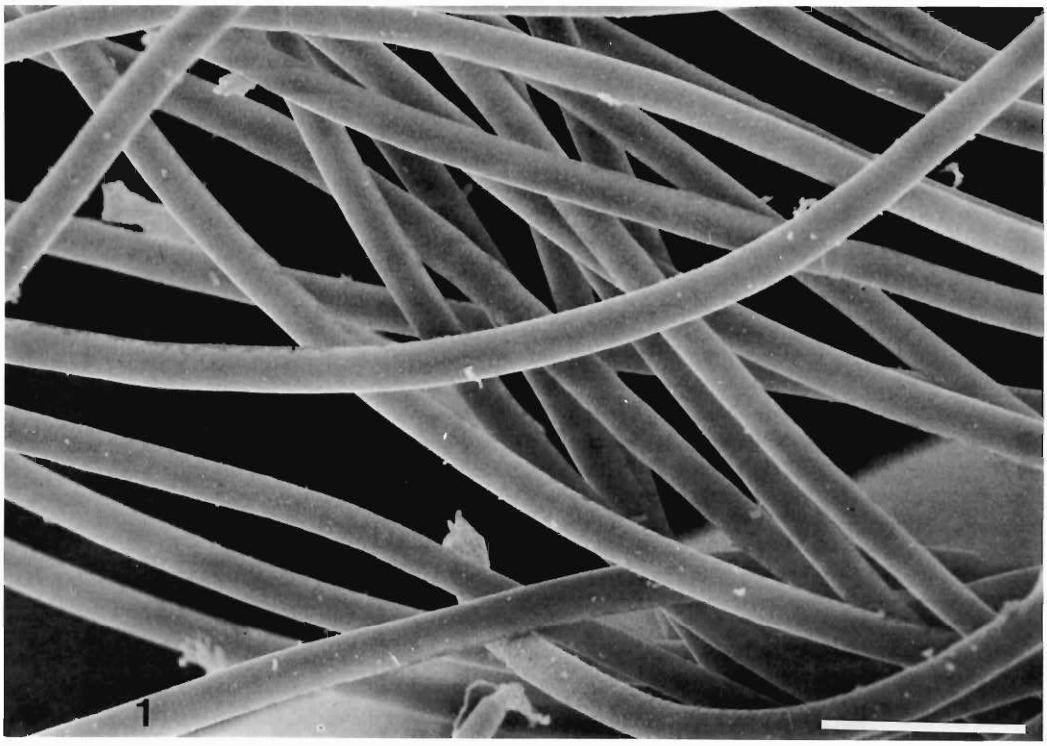
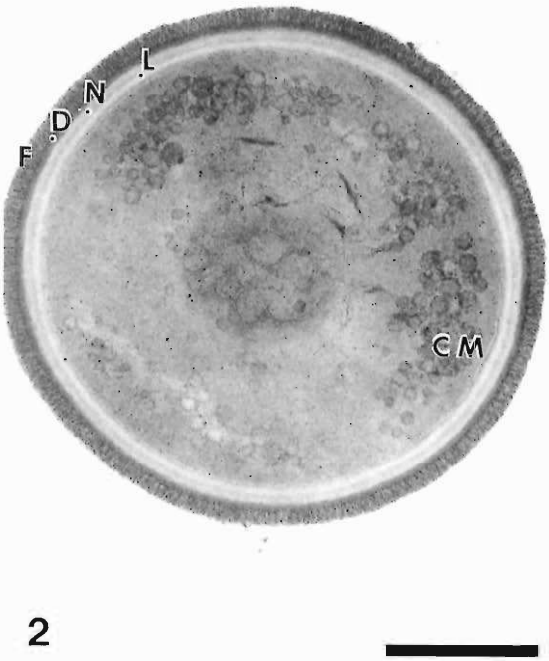
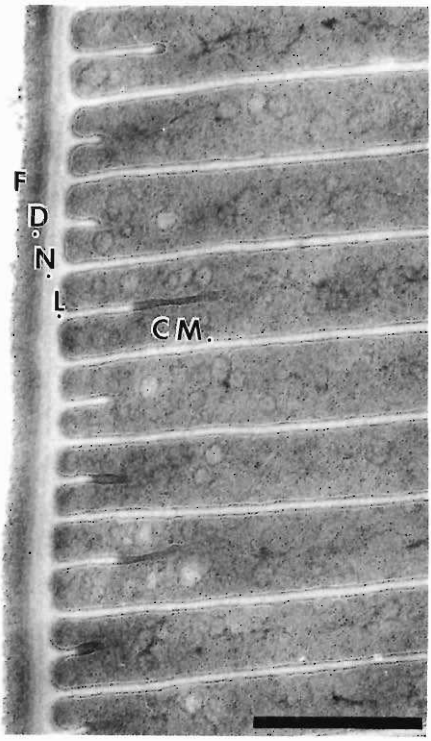
Figures 1–3. Electron microscope views of bacteria associated with cyathostomid nematodes. 1. SEM of the aerial trichome-forming bacteria close to the cyathostome cuticle. The continuous smooth-walled nature of the filaments is evident. Bar = 10 μm. 2. TEM showing the cell-wall structure of a filament in cross-section. The various layers are indicated as follows: F = outer fibrillar layer, D = dense layer, N = nonmembranous layer, L = electron-lucent space (see text), CM = cytoplasmic membrane. Bar = 0.5 μm. 3. TEM of longitudinal section of a filament showing the cell-wall layers as well as the nature of septation. The CM associated with the formation of discs is clearly visible. The outer layers (F–L) of the cell wall are free from indentations. Bar = 0.5 μm.

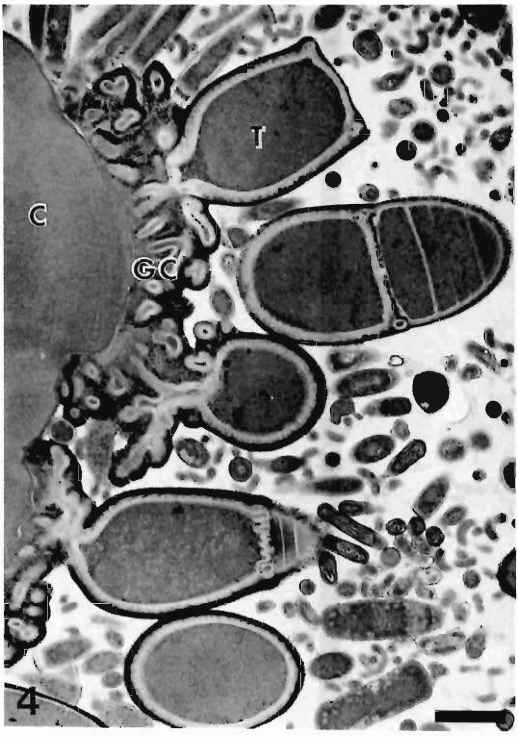
Figures 4–7. TEM of filaments of bacteria associated with cyathostomid nematodes. 4. Multicellular filaments portraying their initial growth phases. Note the growth complex (GC) and its fibrillar nature, the initial barrel-shaped thallus (T) anchored in the GC via a number of roots, or fused branches of the GC giving rise to the thallus. C = cuticle of cyathostome. Bar = 1 μm. 5. Aerial filament showing further internal growth of the thallus with the addition of further cell units (discs). Bar = 1 μm. Inset: Club-shaped structures observed between thallus and first cell. Bar = 1 μm. 6. Filament showing further development in growth with secondary septa at different stages of development growing simultaneously. Bar = 1 μm. 7. A long septate filament showing more stages of secondary annular ingrowth of the CM in various cell units. Bar = 1 μm.

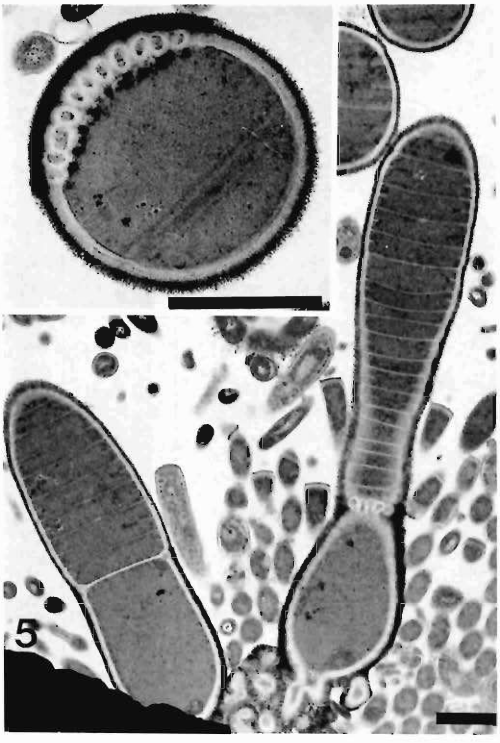
Figures 8–13. TEM of filaments of bacteria associated with cyathostomid nematodes. 8. Longitudinal section showing extensive internal CM ingrowth resulting in numerous thin discs (macrogonidia). Bar = 1 μm. 9. Longitudinal section with more advanced division: septation occurs perpendicular to previous transverse annular ingrowth. Bar = 1 μm. 10. Result of previous 2 directions of septation: spherical or ovoid-shaped elements (microgonidia) are formed giving rise to a multicellular filament. Bar = 1 μm. 11. Cross-section equivalent to the stage in Figure 10. Bar = 1 μm. 12. The last developmental phase: it suggests eventual release of the microgonidia. Bar = 1 μm. 13. Cross-section equivalent to the stage in Figure 12. Note the appearance of fibrillar components on some cells. Bar = 1 μm.

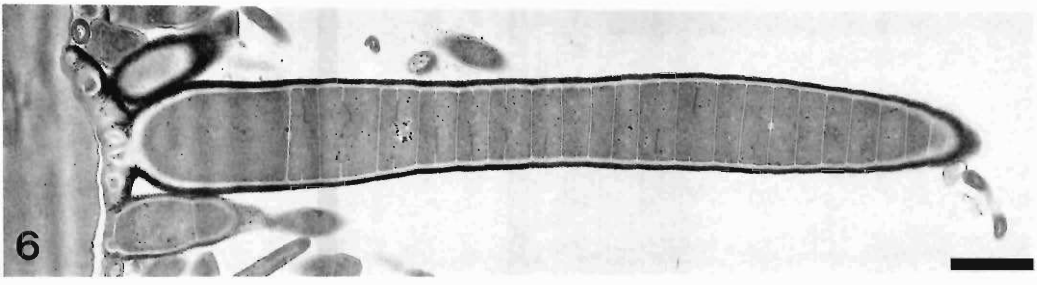


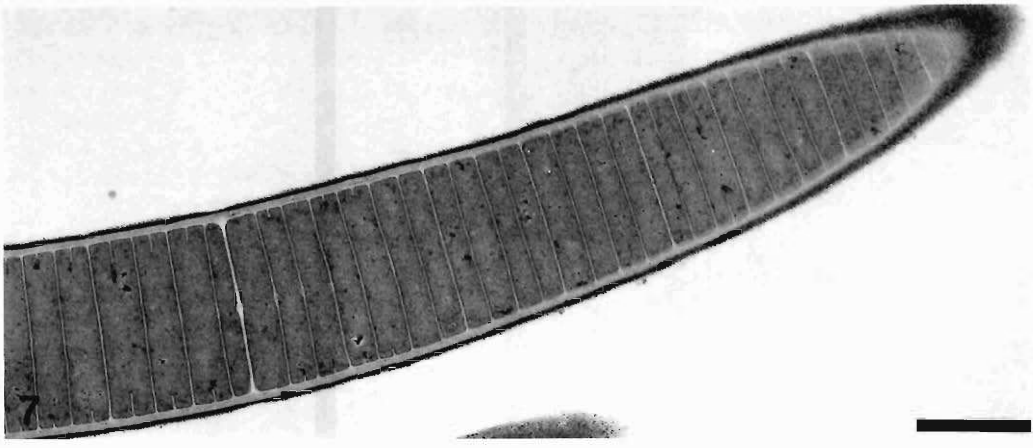


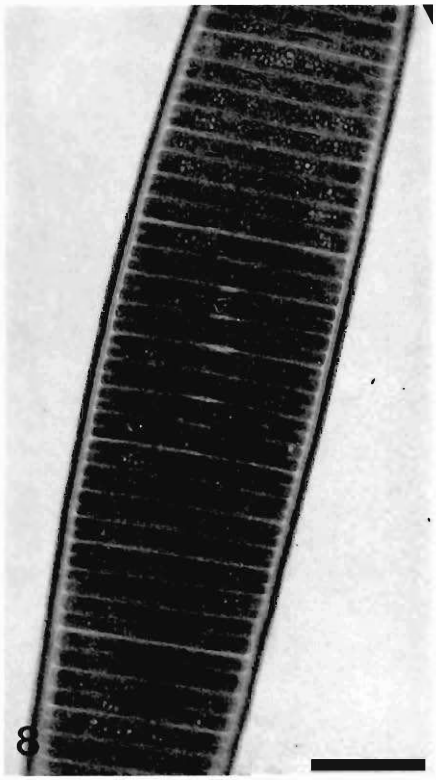


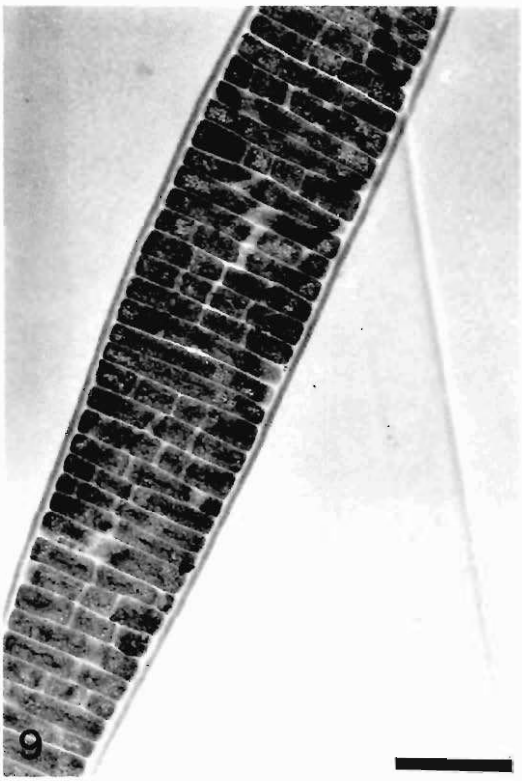


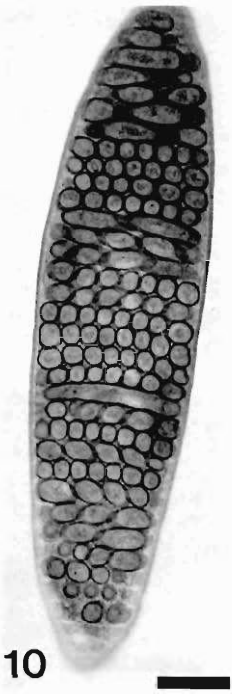


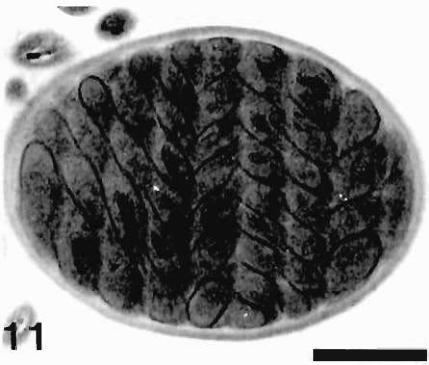


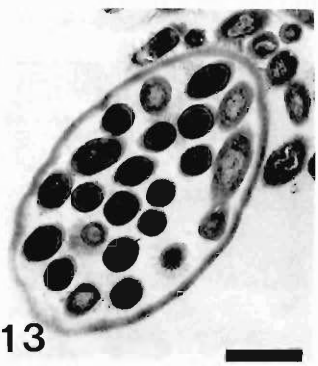


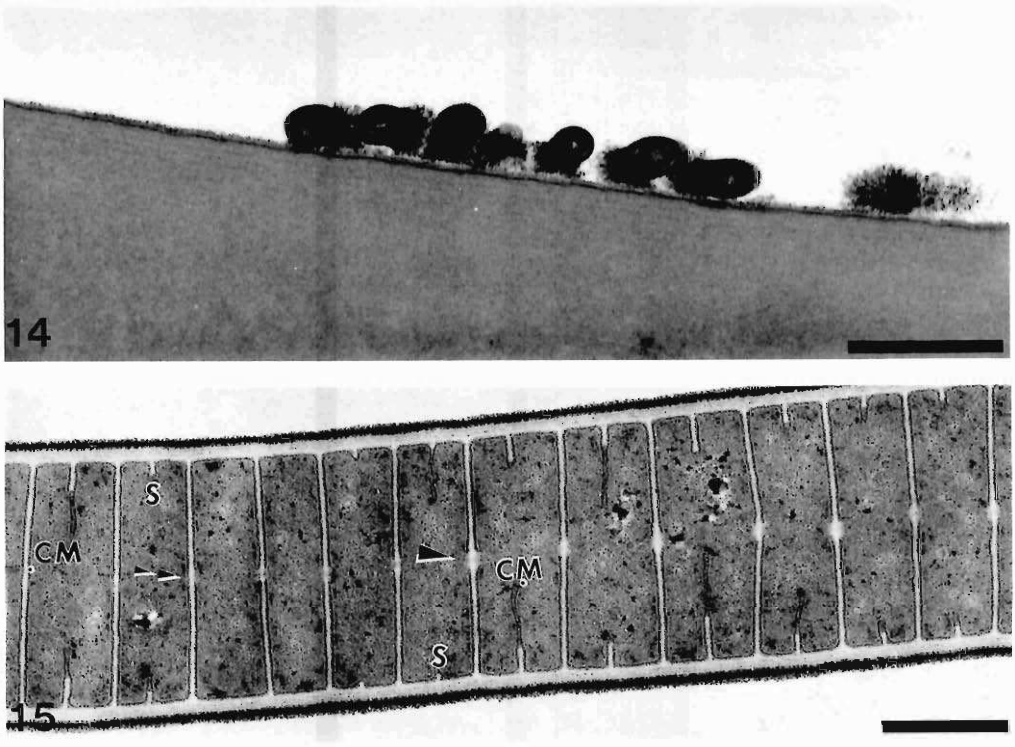












Figures 14, 15. TEM of bacteria of cyathostomes. 14. Microgonidia apparently attaching to the cuticle. Bar = 1 μm. 15. The process of septation in more detail. S = site of septa formation. Note the inward growing CM skirting the septa, the joining of opposing CM in club-shaped (bulbous) structures (arrow), and their fusion (double arrows). Bar = 1 μm.

tures similar to those in many of the other microorganisms are also used for adherence (Els and Krecek, 1990), a growth complex such as that noted here by aid in propagation in this habitat and colonization of the cuticular surface of the cyathostome.

Although RR staining indicates some polysaccharide content, the exact composition and function of the growth complex has not been established. Cauliflowerlike vegetative colonies that form endospores have been described for the genus *Pasteuria* by Sayre and Starr (1989). The vegetative complex of the present study differs morphologically from that of *Pasteuria* and does not appear to share any ultrastructural characteristics.

The sequence of events that follows the release of daughter cells (microgonidia) from the mature filaments, their transformation, and the structure and function of the growth complex is incomplete. The cell-wall structure of the mature filament (Figs. 2, 3) differs from that of the microgonidia (Figs. 10, 12, 13), suggesting that there may be a process of transformation of the wall of the latter during the stages of attachment and development (Figs. 4, 5, 14). It is possible, as with cyanobacteria, that synthesis of the fibrous wall layer is repressed during septation. Also, it is possible that at the time of their release microgonidia may possess walls containing only peptidoglycan and inner membrane layers (Rippka et al., 1979) or that synthesis of the fibrils in the outer wall may accompany release of the microgonidia (Fig. 13). Microgonidia do not appear to resemble spores (Chase and Erlandsen, 1976) nor do the filaments that they form resemble other sporulating filamentous organisms (Bracke et al., 1979; Savage, 1983).

Although morphological characteristics alone are not sufficient to classify the bacterium described here, the developmental stages resemble those demonstrated in other studies of trichomes. These stages correspond to the properties given by Trentini (1981, 1986) that a completed cell unit within the actively growing trichome shows the growth of 1 or more devel-

oping septa and results in new cells and an extension of the trichome length. These cells are usually closely appressed and wider than they are long. Previous reports (Krecek et al., 1987b; Mackie et al., 1989) suggested that filaments resembling those of the present study may belong to the family *Arthromitus* in the order Caryophanales (Peshkoff and Marek, 1973; Trentini, 1981, 1986). Caryophanales is a group of typical filamentous segmented sporeformers (e.g., *Arthromitus* Leidy). Members belonging to *Arthromitus* are trichomes that are observed attached to the intestinal walls of insects and tadpoles (Davis and Savage, 1974). Several trichome-forming bacteria occurring in the alimentary tract of animals have been reported to form endospores but have not been obtained in pure culture (Savage, 1983). The bacterium described here did not exhibit evidence of such endospores.

The microorganisms discussed in this paper may comprise a small part only of the community existing in the zebra's hindgut. In one population, almost 70% of the female cyathostomes exhibited filamentous bacteria attached to their posterior extremities (Krecek et al., 1994). The bacterium in the present study has developed an adaptation for its survival in the production of the numerous daughter cells instead of single endospores. The dispersal of these cells may be the means by which these organisms ensure survival by colonizing a highly specific niche restricted to a precise region on selected cyathostomes.

Further study of this microbial community–cyathostome relationship may aid in our understanding of these bacteria and their function in the zebra hindgut environment. Light microscopical studies suggest that colonization of these filamentous microorganisms is in the vulvar region of female cyathostomes and not in the anal, oral, and excretory orifices (Krecek et al., 1994). The role that the presence of the microorganisms at the vulva bears (i.e., hinders the reproductive activities of the female) has yet to be determined.

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